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Selective substrates and inhibitors for kallikrein-related peptidase 7 (KLK7) shed light on KLK proteolytic activity in the stratum corneum

Simon J. de Veer[1,2,3], Laetitia Furio[2,3], Joakim E. Swedberg[4], Christopher A. Munro[1], Maria Brattsand[5], Judith A. Clements[1,6], Alain Hovnanian[2,3,7], Jonathan M. Harris[1,*]

[1]Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane QLD 4059, Australia

[2]Laboratory of Genetic Skin Diseases, INSERM UMR 1163 and Imagine Institute of Genetic Diseases, 75015 Paris, France

[3]Université Paris V Descartes – Sorbonne Paris Cité, 75006 Paris, France

[4]Institute for Molecular Bioscience, The University of Queensland, Brisbane QLD 4072, Australia

[5]Department of Medical Biosciences, Pathology, Umeå University, 901 87 Umeå, Sweden

[6]Australian Prostate Cancer Research Centre, Translational Research Institute, Brisbane QLD 4102, Australia

[7]Department of Genetics, Necker Hospital for Sick Children, 75015 Paris, France

Short title: KLK activity in the stratum corneum

*Corresponding author:

A/Prof. Jonathan Harris

Institute of Health and Biomedical Innovation, Queensland University of Technology

60 Musk Ave, Kelvin Grove QLD 4059, Brisbane, Australia.

E-mail: j2.harris@qut.edu.au

Tel: +61 7 3138 6186, Fax: +61 7 3138 6030

Abbreviations: AD, atopic dermatitis; EGFR, epidermal growth factor receptor; IL, interleukin; KLK, kallikrein-related peptidase; LEKTI, Lympho-epithelial Kazal-type-related inhibitor; NS, Netherton syndrome; pNA, *para*-nitroanilide; SFTI-1, sunflower trypsin inhibitor-1; SPINK, serine protease inhibitor Kazal-type.

ABSTRACT

Proteases have pivotal roles in the skin's outermost layer, the epidermis. In the stratum corneum, serine proteases from the kallikrein-related peptidase (KLK) family have been implicated in several key homeostatic processes, including desquamation. However, the precise contribution of specific KLKs to each process remains unclear. To address this, we used a chemical biology approach and designed selective substrates and inhibitors for KLK7, the most abundant KLK protease in the stratum corneum. The resulting KLK7 inhibitor is the most potent inhibitor of this protease reported to date (K_i = 140 pM), and displays at least 1,000-fold selectivity over several proteases that are related by function (KLK5 and KLK14) or specificity (chymotrypsin). We then used substrates and inhibitors for KLK5, KLK7 and KLK14 to explore the activity of each protease in the stratum corneum using casein zymography and an *ex vivo* desquamation assay. These experiments provide the most detailed assessment of each KLK's contribution to corneocyte shedding in plantar stratum corneum, revealing that inhibition of KLK7 alone is sufficient to block shedding, while KLK5 is also a major contributor. Collectively, these findings unveil chemical tools for studying KLK activity and demonstrate their potential for characterizing KLK biological functions in epidermal homeostasis.

Key words: chemical biology; desquamation; proteolysis; sunflower trypsin inhibitor-1

INTRODUCTION

Proteases and their inhibitors are now widely recognized as major contributors to epidermal homeostasis. From the early stages of keratinocyte differentiation, proteases are essential for regulating the activity of key signaling molecules, including Notch and EGFR ligands (Mascia et al., 2012). As differentiation proceeds, proteases continue to drive a range of critical processes throughout the epidermis, including in the stratum corneum where they influence cohesion, hydration and acidity by cleaving corneodesmosomes and degrading filaggrin (Ovaere et al., 2009). Considering the importance of these functions, and that proteolytic cleavage is generally irreversible, it is not surprising that altering the activity of a given protease or protease inhibitor can have dramatic effects on the structure and function of the epidermis. Indeed, a growing number of Mendelian genodermatoses have been identified where the defective gene encodes a protease or protease inhibitor, see (de Veer et al., 2014) for recent review. Moreover, it is becoming increasingly apparent that proteases work cooperatively, including with other enzymes, to carry out their biological functions, either by sharing common substrates, deactivating inhibitors for additional proteases or by activating other proteases or enzymes. Accordingly, there has been longstanding interest in delineating the specific roles of individual proteases as a step towards unravelling the different threads that define how proteolysis intertwines with skin homeostasis and disease.

An important family of proteases in the granular and cornified layers of the epidermis are the kallikrein-related peptidases (KLKs). KLK7 (previously named stratum corneum chymotryptic enzyme) was the first KLK protease detected in stratum corneum extracts in active form (Egelrud and Lundstrom, 1991, Hansson et al., 1994), followed by KLK5 (stratum corneum tryptic enzyme) (Brattsand and Egelrud, 1999, Ekholm et al., 2000), KLK14 (Stefansson et al., 2006) and KLK8 (Eissa et al., 2011). Several additional KLKs have been identified in epidermal extracts by ELISA (Komatsu et al., 2006), but their activity has not yet been established. KLK proteolytic activity in the epidermis is controlled by several mechanisms. First, KLKs are expressed as inactive zymogens that require cleavage of an N-terminal pro-peptide to become active (Brattsand et al., 2005). Second, they are regulated by protease inhibitors, including Lympho-epithelial Kazal-type-related inhibitor

(LEKTI), serine protease inhibitor Kazal-type 6 (SPINK6) and SPINK9, that block the KLK active site and prevent substrate binding (Brattsand et al., 2009, Deraison et al., 2007, Meyer-Hoffert et al., 2010, Meyer-Hoffert et al., 2009). Third, their activity is influenced by the biochemical environment, including pH (Hachem et al., 2003), divalent ions (Goettig et al., 2010) and cholesterol sulfate (Sato et al., 1998). Collectively, KLKs are best known for their involvement in desquamation where they promote the shedding of superficial corneocytes by degrading corneodesmosomal cadherins: desmoglein 1, desmocollin 1 and corneodesmosin (Caubet et al., 2004, Lundstrom and Egelrud, 1990, Lundstrom et al., 1994). More recently, the discovery of new putative substrates for KLK proteases has suggested that they participate in other key processes and pathways beyond desquamation. KLK proteases have been shown to activate cell surface receptors that stimulate inflammation, including protease-activated receptor-2 (Oikonomopoulou et al., 2006, Stefansson et al., 2008), to contribute to antimicrobial defense by processing cathelicidin peptides (Yamasaki et al., 2006) and to influence the step-wise maturation of filaggrin (Sakabe et al., 2013).

Several of the biological functions described above feature prominently in skin diseases that involve dysregulated KLK activity. In Netherton syndrome (NS), the major inhibitor of KLKs in the epidermis, LEKTI, is absent due to mutations in the *SPINK5* gene (Chavanas et al., 2000). This causes a significant imbalance between proteolysis and inhibition, and results in a severe skin disease that involves detachment of the stratum corneum due to premature degradation of corneodesmosomes (Descargues et al., 2005, Descargues et al., 2006, Yang et al., 2004), defective lipid processing (van Smeden et al., 2014), accelerated filaggrin degradation (Descargues et al., 2005, Hewett et al., 2005), and persistent skin inflammation with severe allergies (Briot et al., 2009, Furio et al., 2014, Hannula-Jouppi et al., 2014). Skin barrier defects and dysregulated inflammation are also pivotal in atopic dermatitis (AD) (Elias and Wakefield, 2014), and increased levels of several KLKs, including KLK5 and KLK7, have been identified in the stratum corneum of AD patients (Komatsu et al., 2007). Recent studies have shown that Th2 cytokines, including IL-4 and IL-13, stimulate expression of KLK7 in cultured keratinocytes, suggesting a direct link between inflammation in AD and levels of KLK7 (Hatano et al., 2013, Morizane et al., 2012). KLKs are also implicated in skin diseases that feature

impaired desquamation, where KLK activity is diminished due to defective KLK transport, reduced zymogen activation or changes in the biochemical environment. These abnormalities occur in Harlequin ichthyosis (Kelsell et al., 2005, Thomas et al., 2009), autosomal recessive ichthyosis with hypotrichosis (Basel-Vanagaite et al., 2007, Sales et al., 2010) and X-linked ichthyosis (Elias et al., 2014, Webster et al., 1978), respectively.

Although KLKs are collectively associated with key roles in epidermal homeostasis, at present, the precise contribution of each protease to each function remains unclear. For example, while KLK5, KLK7 and KLK14 have all been shown to be active in the stratum corneum and to cleave corneodesmosomal cadherins, each protease's specific contribution to desquamation has not been clearly defined. Similarly, in skin diseases such as NS, several KLKs are dysregulated and capable of eliciting the major hallmarks of the disease, such as degrading desmosomes or filaggrin, and stimulating inflammation. Recent studies investigating new animal models, including transgenic KLK5 mice (Furio et al., 2014) and KLK5 deficient mice (Furio et al., 2015), have made appreciable progress toward understanding the importance of KLK5 in the epidermis. These biological approaches could be complemented by developing chemical tools (substrates and inhibitors) that can be used to explore the activity of separate proteases in different assays. In recent work, we designed peptide substrates and inhibitors for trypsin-like KLKs, KLK5 (de Veer et al., 2015a, de Veer et al., 2016) and KLK14 (de Veer et al., 2012, de Veer et al., 2015b). In this study, we focused on KLK7, a chymotrypsin-like protease that is the most abundant KLK in the stratum corneum (Komatsu et al., 2006). We design synthetic substrates and high affinity inhibitors for KLK7, and use these chemical tools, together with substrates and inhibitors for KLK5 and KLK14, to explore KLK proteolytic activity in plantar stratum corneum and the contribution of each protease to corneocyte shedding in an *ex vivo* model.

RESULTS

Identifying new KLK7 substrates using a synthetic peptide library screen

KLK7 is widely regarded to be an important protease in the stratum corneum, but there are relatively few chemical tools that are available for studying its biological activity. To identify synthetic substrates for KLK7, we produced and screened a library of 120 peptides, each comprising a four amino acid peptide segment conjugated to a colorimetric reporter (*para*-nitroanilide, pNA) at the C-terminus (Figure 1a). Substrate sequences included in the library were based on KLK7 subsite specificity data that was generated by screening a positional scanning library (Debela et al., 2006). KLK7 has chymotrypsin-like specificity and generally cleaves substrates after a Tyr or Phe residue. Across the peptide library, substrates cleaved after Tyr were generally favored compared to those containing Phe (Figure 1b), indicating a preference for Tyr at P1. Additionally, Leu and Val were preferred at P2 over Thr and Asn, sequences containing His at P3 were favored over Gln and Thr, and Lys was clearly preferred at P4. The optimal KLK7 substrate identified was KHLY, followed by KTLY and KHLF (Figure 1b).

For comparison, the substrate library was also screened against chymotrypsin, which also cleaves substrates after Tyr or Phe but has a broader extended specificity. Indeed, 36/120 sequences were cleaved by chymotrypsin at more than half the rate of the optimal substrate (FTTY, Figure 1c), compared to only 6/120 by KLK7. Despite this, all substrates containing Lys at P4 were cleaved by chymotrypsin at relatively low rates, such that the five sequences most preferred by KLK7 (KHLY, KTLY, KHLF, KQLY and KHVY) were all disfavored by chymotrypsin. Thus, while KLK7 and chymotrypsin have similar primary (P1) specificity, the two proteases display differences in their extended specificity. We also determined kinetic constants (k_{cat} and K_M, Table 1) to compare the new optimal KLK7 substrate (KHLY-pNA) to the commercially available substrate that is commonly used to detect KLK7 activity in skin extracts (MeO-Suc-RPY-pNA) (Franzke et al., 1996, Komatsu et al., 2007, Voegeli et al., 2009). These analyses revealed that cleavage of KHLY-pNA by KLK7 produced a 15-fold lower K_M value (indicating higher affinity) and 10-fold higher k_{cat} value (indicating faster turnover rate) than MeO-Suc-RPY-pNA, which resulted in a 150-fold improvement in catalytic

efficiency (k_{cat}/K_M). Moreover, MeO-Suc-RPY-pNA is a more efficient substrate for chymotrypsin than KLK7 by two orders of magnitude (Franzke et al., 1996, Schellenberger et al., 1991). However, this effect was reversed for KHLY-pNA, which was a more efficient KLK7 substrate (comparing k_{cat}/K_M values). Interestingly, replacing P4 Lys with Gln (QHLY-pNA) restored efficient cleavage by chymotrypsin (Table 1), indicating that the P4 Lys residue was important for achieving KLK7 selectivity.

Engineering high affinity KLK7 inhibitors using specificity data from substrate and inhibitor library screens

The peptide substrates for KLK7 interact with the protease active site in a similar manner to the binding loop of many protease inhibitors, including LEKTI. Accordingly, highly favored substrate sequences can be substituted into the binding loop of a naturally occurring inhibitor to re-direct the inhibitor's activity towards a new target protease (Swedberg et al., 2010). We used this strategy to develop KLK7 inhibitors based on sunflower trypsin inhibitor-1 (SFTI-1), a 14 amino acid cyclic peptide that is found in sunflower seeds and is a high affinity inhibitor of trypsin (K_i < 0.1 nM) (Luckett et al., 1999). First, we substituted the sequence of the optimal KLK7 substrate (KHLY) into the P4, P2 and P1 residues of SFTI-1 (P3 Cys was not replaced with His in order to preserve the inhibitor's intramolecular disulfide bond) (Swedberg et al., 2009), and replaced Asp14 with Asn (variant 2, Table 2). Whereas SFTI-1 showed minimal activity against KLK7 (IC_{50} > 10 μM), substituting a favored substrate sequence into the inhibitor's binding loop yielded a potent KLK7 inhibitor (K_i = 2.5 nM). Similarly, substituting the P1 Tyr residue with Phe produced an effective KLK7 inhibitor (K_i = 4.0 nM, variant 3), with the decrease in activity consistent with KLK7's preference for KHLY-pNA compared to KHLF-pNA (Table 1). As KLK7 is the only known protease in the stratum corneum to have chymotrypsin-like specificity, we assessed the selectivity of the engineered inhibitors against chymotrypsin. However, neither inhibitor showed suitable selectivity (Table 2), with variant 3 showing weaker activity against chymotrypsin than variant 2.

In a recent study, we showed that substituting a further residue in the inhibitor's binding loop (P2′) can improve the activity and selectivity of engineered SFTI variants (de Veer et al., 2015b). Comparing the P2′ specificity profiles for KLK7 and chymotrypsin (de Veer et al., 2015b) indicated that substituting P2′ Ile (variants 1-3) with Glu, Ser or Asn could decrease the activity of variant 3 against chymotrypsin, and thus improve its selectivity for KLK7. Assessing the new inhibitors (variants 4-6) against KLK7 and chymotrypsin revealed that all three substitutions provided considerable increases in selectivity (Table 2). Substituting P2′ Ile with Asn (variant 6) produced the most potent KLK7 inhibitor (K_i = 140 pM), which represented an improvement in activity of 28-fold compared to variant 3, while also providing over 1,000-fold selectivity over chymotrypsin. Interestingly, Asn is also present at P2′ in eight of the fifteen LEKTI domains (Magert et al., 1999).

Examining KLK proteolytic activity in human stratum corneum extracts using synthetic substrates and inhibitors

By designing a potent and selective KLK7 inhibitor, we had now developed inhibitors for the three KLK proteases most strongly linked to desquamation: KLK5 (de Veer et al., 2016), KLK7 (this study) and KLK14 (de Veer et al., 2015b). First, we examined proteolytic activity in plantar stratum corneum extracts using casein zymography. Here, casein gels were treated post-electrophoresis with SFTI-KLK5, SFTI-KLK7 or SFTI-KLK14 (see Table 2 for inhibitor sequences and K_i values) to block the activity different KLKs, as well as a fourth inhibitor that potently inhibits all three KLKs, SFTI-KLK5/7/14 (de Veer et al., 2015b). Casein gels incubated without inhibitor revealed five bands of proteolytic activity in stratum cornum extracts between ~30 kDa and 20 kDa (Figure 2a). Including SFTI-KLK7 in the activity buffer led to inhibition of the lower two bands, whereas the upper three bands were inhibited by SFTI-KLK5. Although each inhibitor blocked several bands of proteolytic activity, previous studies have shown that active KLK5 isolated from plantar stratum corneum yields three bands on non-reducing SDS-PAGE (Brattsand and Egelrud, 1999) and active KLK7 yields two bands (Egelrud, 1993), with the known migration pattern for KLK5 and KLK7 matching the inhibition profile for SFTI-KLK5 and SFTI-KLK7. Interestingly, none of the bands of proteolytic

activity appeared to be inhibited by SFTI-KLK14, whereas including SFTI-KLK5/7/14 in the activity buffer resulted in inhibition of all five bands of proteolytic activity.

To validate our findings from stratum corneum extracts, we assessed the ability of SFTI-KLK5, SFTI-KLK7 and SFTI-KLK14 to inhibit recombinant KLKs in casein zymography. Gels incubated without inhibitor displayed one major and one minor band of proteolytic activity for recombinant KLK7 and KLK14 (Figure 2b). Recombinant KLK5 produced three major bands and one minor band, and previous studies have shown that these correspond to glycosylation variants (Michael et al., 2005). Including SFTI-KLK7 or SFTI-KLK14 in the activity buffer enabled complete inhibition of KLK7 or KLK14, respectively, with no substantial off-target inhibition (Figure 2b). This observation confirmed that 5 μM SFTI-KLK14 is sufficient to inhibit KLK14 in gel zymography assays. It also provided further evidence that none of the bands of activity seen in Figure 2a corresponded to KLK14, as although KLK14 shows similar electrophoretic mobility to KLK7 (Figure 2b), in stratum corneum extracts, we did not observe inhibition of either of the lower two bands after incubation with SFTI-KLK14, nor did we observe any remaining activity after incubation with SFTI-KLK7. SFTI-KLK5 produced complete inhibition of KLK5, but with partial off-target inhibition of KLK14. This was not unexpected given that SFTI-KLK5 only showed 10-fold selectivity over KLK14 in kinetic assays (Table 2), but importantly, no off-target inhibition of KLK7 was observed, either by zymography or in kinetic assays. Additionally, by examining gels treated with SFTI-KLK14, we could verify that none of the bands inhibited by SFTI-KLK5 in stratum corneum extracts corresponded to off-target inhibition of KLK14.

Given that active KLK5 and active KLK7 were identified in plantar stratum corneum extracts using zymography, we also examined whether synthetic peptide substrates could be used to detect KLK5 or KLK7 activity in these samples. For these assays, we used the optimal substrate for KLK7 (KHLY-pNA, Table 1). However, we did not use the optimal substrate for KLK5, Ac-YRSR-pNA (de Veer et al., 2016), as it contains a second Arg residue that could serve as an internal cleavage point, and chose to use Ac-YASR-pNA. Using 500 ng of stratum corneum protein extract, we could readily detect KLK7 activity, with the increase in absorbance at 405 nm (corresponding to substrate cleavage)

remaining essentially linear over the 24 hour time course (Figure 2c). Moreover, cleavage of KHLY-pNA could be completely blocked by including SFTI-KLK7 in the assay buffer, indicating that substrate cleavage could be attributed to KLK7. Cleavage of Ac-YASR-pNA was also detected, with 50% inhibition using 2 µM SFTI-KLK5 and 65% inhibition using 5 µM SFTI-KLK5 (Figure 2d). As this concentration of inhibitor was sufficient to block KLK5 activity in zymography experiments, these data suggest that Ac-YASR-pNA might also be cleaved by another protease present in stratum corneum extracts.

Defining the contribution of separate KLK proteases to *ex vivo* corneocyte shedding

Degradation of corneodesmosomes to promote corneocyte shedding was the first major role described for KLK proteases in the epidermis. Therefore, we tested inhibitors for KLK5, KLK7 and KLK14 in the *ex vivo* desquamation assay described by Lundstrom and Egelrud (1988) to provide insight into the contribution of each protease to corneocyte shedding. Treating stratum corneum samples with SFTI-KLK5 partially blocked corneocyte shedding (48% inhibition at 5 µM), indicating that KLK5 is a direct contributor to corneocyte shedding in this assay. Although this inhibitor is not strictly selective for KLK5, at the very least, these data reflect the contribution of KLK5 and possibly another trypsin-like protease. We can potentially exclude input from KLK14, as SFTI-KLK14 produced no effect at 5 µM. By contrast, treatment with SFTI-KLK7 produced a dramatic reduction in corneocyte shedding (95% inhibition at 2.5 µM) that was essentially maintained at a lower concentration of inhibitor (91% inhibition at 1 µM). As SFTI-KLK7 is highly selective for KLK7 (Table 2, Figure 2), these data suggest that KLK7 activity is required for *ex vivo* corneocyte shedding in plantar stratum corneum. Finally, treating samples with a single inhibitor that targets multiple KLKs, SFTI-KLK5/7/14, produced the most pronounced inhibition of corneocyte shedding (99% inhibition at 2.5 µM).

DISCUSSION

In this study, we designed selective chemical tools for studying the activity of KLK7, and used these molecules, together with substrates and inhibitors for KLK5 and KLK14, to explore KLK proteolytic activity in human stratum corneum. The KLK7 substrate that we describe is over 150-fold more efficient than the commonly used commercial substrate, MeO-Suc-RPY-pNA, and the optimal KLK7 inhibitor that we designed is the most potent inhibitor of this protease reported to date, showing more than three orders of magnitude in selectivity over KLK5, KLK14 and chymotrypsin. Subsequently applying different inhibitors and substrates to stratum corneum extracts or tissue samples allowed us to characterize KLK proteolytic activity in the stratum corneum in greater depth, which we demonstrated by dissecting the specific contribution of KLK5, KLK7 and KLK14 to *ex vivo* corneocyte shedding.

One of the key findings to emerge in this study is that *ex vivo* corneocyte shedding in plantar stratum corneum requires the activity of KLK7. In a physiological context, desquamation is widely regarded to involve several proteases that act co-operatively (Borgono et al., 2007, Furio and Hovnanian, 2014, Ovaere et al., 2009), but until now, it was not possible to define the individual contributions of KLK5, KLK7 and KLK14. Using engineered inhibitors that strictly discriminate between trypsin-like (KLK5 and KLK14) and chymotrypsin-like KLKs (KLK7), we showed that selective inhibition of KLK7 was sufficient to block corneocyte shedding. KLK7 is the most abundant KLK protease in the stratum corneum (Komatsu et al., 2006), and has been shown to directly cleave desmocollin 1 and corneodesmosin *in vitro* (Caubet et al., 2004), indicating a prominent role for KLK7 in desquamation. However, previous studies examining corneocyte shedding in plantar stratum corneum identified that both desmoglein 1 (Lundstrom and Egelrud, 1990) and corneodesmosin (Lundstrom et al., 1994) were cleaved in shed corneocytes. Desmoglein 1 is not regarded to be a substrate for KLK7 (Caubet et al., 2004), suggesting that additional proteases are also involved in *ex vivo* corneocyte shedding. Indeed, we identified that inhibitors for KLK5 were also able to decrease corneocyte shedding, and KLK5 has been reported to cleave desmoglein 1 *in vitro* (Caubet et al.,

2004). Thus, while corneocyte shedding could be blocked by inhibiting KLK7, our data more broadly demonstrate that desquamation involves both trypsin-like and chymotrypsin-like proteases.

A further consideration when assessing the contribution of KLKs to epidermal homeostasis is the influence of protease activation cascades. Both KLK5 and KLK7 are expressed as inactive zymogens and only become active once their N-terminal pro-peptide has been cleaved. Currently, the main candidates for activating pro-KLK5 and pro-KLK7 in the stratum corneum include KLK5 (Brattsand et al., 2005), matriptase (Sales et al., 2010) and potentially mesotrypsin (Miyai et al., 2014). These proteases have the capacity to indirectly influence desquamation, as seen in autosomal recessive ichthyosis with hypotrichosis, where a predicted loss of function missense mutation at the homozygous state in the *ST14* gene that encodes matriptase leads to hyperkeratosis and impaired corneodesmosome degradation (Basel-Vanagaite et al., 2007). In the *ex vivo* model used in this study, tissue samples were derived from the uppermost portion of the stratum corneum, and our analyses using casein zymography and synthetic peptide substrates indicated that considerable pro-KLK activation had already occurred. Thus, inhibition of proteases such as KLK5 may have a greater effect in experiments where inhibitor treatment is able to capture the protease's direct and indirect contributions.

An unexpected finding in this study was the lack of activity that could be attributed to KLK14 in casein zymography experiments and *ex vivo* desquamation assays. Active KLK14 has previously been purified from human plantar stratum corneum extracts and suggested to contribute significantly to trypsin-like proteolytic activity in this tissue (Stefansson et al., 2006). However, there are several factors that need to be considered when comparing the findings from both studies. First, trypsin-like activity was detected in the previous study using the synthetic substrate, S-2288, which is a particularly poor KLK5 substrate (k_{cat}/K_M = 57 M^{-1} s^{-1}) and is cleaved by KLK14 with 180-fold higher efficiency (Brattsand et al., 2005). Thus, cleavage of S-2288 will likely underestimate KLK5 activity in the extract, and consequently, may overestimate the contribution of other proteases, including KLK14. Second, it is important to note that KLK14 activity detected by Stefansson and colleagues was in samples prepared from 10 g starting material that was refined by two chromatography steps,

whereas our study used crude extracts from less starting material. Given that KLK14 is present at relatively low levels in human stratum corneum (between 0.7% and 1.5% of total trypsin-like KLKs by ELISA) (Komatsu et al., 2006), and that chromatography steps will allow concentration of the target protein, it is possible that we could detect activity corresponding to KLK5 and KLK7 on casein zymography, similar to previous studies, as these proteases are more abundant, but the amount of KLK14 in our extracts was below the detection limit. With this in mind, our findings do not indicate that active KLK14 is absent in the stratum corneum. However, as the *ex vivo* desquamation assay is able to assess the contribution of each protease to corneocyte shedding directly in the stratum corneum, our data suggest that KLK14 does not have a major role in this particular assay.

The chemical tools that we have developed for KLK5, KLK7 and KLK14 are also useful for exploring KLK proteolytic activity in a range of skin diseases. In NS, the absence of LEKTI leads to dysregulation of the KLK proteolytic cascade and affects several proteases, including KLK5, KLK7 and elastase-2 (Bonnart et al., 2010, Descargues et al., 2006). The inhibitors and substrates used in our study have potential for examining the specific contribution(s) of each KLK to key disease mechanisms in NS, as we demonstrated here with *ex vivo* corneocyte shedding. Indeed, our findings from *ex vivo* desquamation assays may suggest that in NS, rapid activation of pro-KLK7 due to uncontrolled KLK5 activity is a more significant event than is currently appreciated. This hypothesis is consistent with recent findings from NS animal models, including transgenic KLK5 mice where increased KLK5 expression in the epidermis leads to elevated KLK7 activity (Furio et al., 2014), and conversely in *Spink5*$^{-/-}$ mice, where deleting KLK5 leads to a dramatic decrease in KLK7 activity and prevents premature degradation of corneodesmosomes (Furio et al., 2015). KLK proteases also attract considerable interest in AD as elevated levels of several KLKs have been detected in the stratum corneum of AD patients (Komatsu et al., 2007). However, whether each KLK is predominantly in zymogen form or active form has not been thoroughly studied. The inhibitors and substrates that we report are well suited for profiling the activity of separate KLKs, and thus, could be used to examine the correlation between KLK expression and KLK proteolytic activity in AD. In addition to serving as research tools in NS and AD, SFTI-based inhibitors that are effective against the major KLKs in each

disease could also see use as therapeutic leads and contribute to restoring epidermal barrier function by blocking dysregulated KLK proteolytic activity.

MATERIALS AND METHODS

Recombinant protease expression

Recombinant pro-KLK5 and pro-KLK7 were expressed in *Pichia pastoris* strain X-33, using previously reported constructs (Brattsand et al., 2009, Stefansson et al., 2008). Recombinant pro-KLK14 was expressed in Sf9 insect cells (*Spodoptera frugiperda*) as previously described (Ramsay et al., 2008, Swedberg et al., 2009). Full details are provided in de Veer et al. (2015a), including methods for pro-KLK purification and activation. Bovine α-chymotrypsin was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

Synthesis of peptide substrates and inhibitors

Peptide-pNA substrates were synthesized on *p*-phenylenediamine derivatized 2-chlorotrityl resin using Fmoc chemistry, as described recently (de Veer et al., 2016). Substrates used to determine kinetic constants or used in stratum corneum activity assays were purified by reverse phase HPLC. Engineered SFTI variants were produced by microwave-assisted solid phase peptide synthesis using recently described protocols (de Veer et al., 2015a) and were purified by reverse phase HPLC.

Kinetic assays

Substrate library screening, active site titration and protease inhibition assays were performed as recently described (de Veer et al., 2016). Briefly, peptide-pNA substrates were adjusted to approximate equimolarity (100 µM) and screened in 96-well low-binding plates against KLK7 or chymotrypsin in 250 µl assay buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 0.005% Triton X-100). Buffer for chymotrypsin assays also included 10 mM $CaCl_2$. Substrate cleavage rates were analyzed by measuring the absorbance at 405 nm over 300 s (reading interval: 10 s) using a microplate spectrophotometer and assays were performed three times in duplicate. Assays for determining substrate kinetic constants (k_{cat} and K_M) were performed in the same way, using a serial dilution of

purified substrate and a constant concentration of protease. Kinetic constants were subsequently determined by non-linear regression (Michaelis-Menten model) in GraphPad Prism 5. To determine the inhibition constant (K_i), KLK7 or chymotrypsin were screened against a serial dilution of inhibitor while the concentration of protease and substrate was fixed (KLK7: KHLY-pNA, chymotrypsin: Ac-GRPY-pNA). K_i was calculated by non-linear regression (Morrison equation) in GraphPad Prism 5 and assays were performed three times in triplicate.

***Ex vivo* desquamation assays**

KLK involvement in corneocyte shedding was examined using the *ex vivo* desquamation assay described by Lundstrom and Egelrud (1988) following guidance from the Queensland University of Technology ethics committee. Cylinders of human plantar stratum corneum were cut using a 4 mm biopsy punch, and first incubated in 500 µl buffer containing 0.01 M sodium phosphate (pH 7.4), 0.14 M NaCl, 0.1% sodium azide and 0.5% Triton X-100 for 4 hours at room temperature. Loosely attached corneocytes were dislodged by vortexing (10 s), then tissue cylinders were incubated in 750 µl activity buffer (0.1 M Tris-HCl pH 8.0, 5 mM EDTA, 0.1 % sodium azide) for 18 hours at 37°C. After vortexing samples (20 s), intact tissue pieces were removed and detached corneocytes were collected by centrifugation (13,000 × *g*, 10 min). The supernatant was aspirated and corneocyte pellets were washed with PBS, centrifuged (as above), then solubilised in 1 M NaOH (350 µl, 60°C, 90 min). Alkali-soluble protein was measured by the Bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Melbourne, Australia) to quantify the number of detached corneocytes. For samples treated with engineered SFTI variants, inhibitors were added to both equilibration and activity buffer.

Preparation of plantar stratum corneum extracts

After biopsies had been cut for *ex vivo* desquamation assays, remaining stratum corneum was finely diced using a scalpel blade and extracted in 1 M acetic acid (1.5 ml) at 4°C for 48 hours (mixing by inversion). The extract suspension was clarified by centrifugation (13,000 × *g*, 30 min, 4°C), the supernatant was recovered, and solvent was evaporated using a Speed-Vac. Dried extracts were

resuspended in H_2O overnight at 4°C (mixing by inversion), then clarified by centrifugation (13,000 × *g*, 30 min, 4°C). Protein content in each sample was quantified by Bradford assay (Biorad).

Gel zymography

Stratum corneum extracts (500 ng per lane) or recombinant KLKs were diluted in non-reducing Laemmli sample buffer and separated by SDS-PAGE using 15% acrylamide gels cast with 0.1% (*w*/*v*) bovine α-casein (Sigma-Aldrich, Sydney, Australia) in the resolving gel. Following electrophoresis, gels were washed with 2.5% (v/v) Triton X-100 (2 × 30 min), then incubated in activity buffer (50 mM Tris-HCl, pH 8.0) for 18 hours at 37°C. For gels treated with KLK inhibitors, inhibitors were included in the final wash step, and in the activity buffer. Gels were stained with Coomassie Brilliant Blue and areas of proteolytic activity were visible as clear bands against a dark background.

Stratum corneum activity assays using synthetic peptide substrates

Stratum corneum protein extracts (500 ng per well) were added to activity buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 0.05% NaN_3, 0.005% Triton X-100) in 96-well low-binding plates. Extracts were next pre-incubated with SFTI-KLK5 (2 µM or 5 µM), SFTI-KLK7 (1 µM or 2.5 µM) or an equal volume of activity buffer (where no inhibitor was added), in a total volume of 200 µl. After 5 min incubation, KHLY-pNA or Ac-YASR-pNA were added (diluted in 50 µl activity buffer), each at a final concentration of 120 µM. Plates were incubated at 37°C, and substrate cleavage was tracked by measuring the absorbance at 405 nm after 0, 2, 4, 6 and 24 hours using a microplate spectrophotometer. Control wells that contained substrate in activity buffer without protein extract were also included, and assays were performed three times in triplicate, each using a separate protein extract.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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TABLES

Table 1. Characterization of synthetic peptide substrates for KLK7

Protease	Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
KLK7	KHLY-pNA	44 ± 1.0	12 ± 0.1	2.7×10^5
KLK7	QHLY-pNA	49 ± 1.6	7.7 ± 0.07	1.6×10^5
KLK7	KHLF-pNA	48 ± 1.4	7.4 ± 0.07	1.5×10^5
KLK7[1]	MeO-Suc-RPY-pNA	650	1.2	1.8×10^3
Chymotrypsin	KHLY-pNA	180 ± 9.4	4.4 ± 0.1	2.4×10^4
Chymotrypsin	QHLY-pNA	86 ± 4.8	40 ± 0.7	4.7×10^5
Chymotrypsin[2]	MeO-Suc-RPY-pNA	-	-	2.8×10^5

[1]Values reported in Franzke et al. (1996)
[2]Values reported in Schellenberger et al. (1991)

Table 2. Inhibition constants (K_i) for KLK7 inhibitors (variants 2-6) and previously reported KLK5 or KLK14 inhibitors used in this study

		K_i		
Variant	**Sequence**[1]	**KLK7**	**Chymotrypsin**	**Fold selectivity**
1 (SFTI-1)	GRCTKSIPPICFPD	> 10,000 nM	252 ± 12 nM	-
2	GKCL**Y**SIPPICFP**N**	2.5 ± 0.2 nM	14 ± 1.0 nM	5.6
3	GKCL**F**SIPPICFP**N**	4.0 ± 0.2 nM	32 ± 1.8 nM	8.0
4	GKCL**F**S**E**PPICFP**N**	3.1 ± 0.1 nM	5,400 ± 230 nM	1,742
5	GKCL**F**S**S**PPICFP**N**	1.4 ± 0.1 nM	943 ± 40 nM	674
6	GKCL**F**S**N**PPICFP**N**	0.14 ± 0.01 nM	170 ± 6.7 nM	1,214
Variant	**Sequence**	**KLK5**	**KLK7**	**KLK14**
SFTI-KLK5[2]	G**Y**C**N**R**S**Y**P**PIC**N**P**N**	5.1 ± 0.2 nM	> 10,000 nM	52 ± 1.4 nM
SFTI-KLK7	GKCL**F**SNPPICFP**N**	> 10,000 nM	0.14 ± 0.01 nM	> 10,000 nM
SFTI-KLK14[3]	G**W**C**I**R**S**K**P**PIC**N**P**N**	> 10,000 nM	> 10,000 nM	7.0 ± 0.5 nM
SFTI-KLK5/7/14[3]	G**T**CT**R**S**N**P**P**IC**N**P**N**	5.2 ± 0.2 nM	0.8 ± 0.05 nM	1.2 ± 0.07 nM

[1]Residues modified from SFTI-1 are highlighted in bold and the P1 residue is underlined. All inhibitors are head-to-tail backbone cyclized and a disulfide bond is formed between Cys3 and Cys11.
[2]Values reported in de Veer et al. (2016)
[3]Values reported in de Veer et al. (2015b)

FIGURE LEGENDS

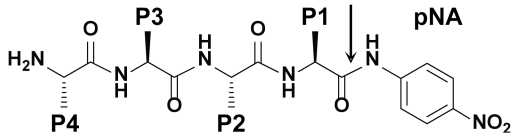
Figure 1. KLK7 substrate specificity analysis. (a) Schematic representation of a peptide-pNA substrate showing the P1, P2, P3 and P4 residues and the *para*-nitroanilide (pNA) reporter. The arrow indicates the peptide bond that undergoes proteolytic cleavage. Heat maps illustrate cleavage rates by (b) KLK7 or (c) chymotrypsin against a library of 120 synthetic peptide substrates. Data are normalized to the optimal substrate for each protease (KLK7: KHLY [24 mOD min^{-1}], chymotrypsin: FTTY [26 mOD min^{-1}]) and are represented using a continuous gradient from 100% (highest rate, green) to 0% (black). Row headings show the P4 residue and column headings show the P3-P2-P1 sequence for each peptide (colored green for each protease's optimal substrate).

Figure 2. Profiling KLK proteolytic activity in plantar stratum corneum using engineered inhibitors and substrates. Engineered SFTI inhibitors were used to block the activity of different KLK proteases in casein zymography assays, either to (a) characterize proteolytic activity in stratum corneum extracts or (b) validate inhibitor selectivity using recombinant proteases. Inhibitors (indicated above each gel) were included in the activity buffer and gels were stained with Coomassie Brilliant Blue to visualize areas of proteolytic activity (clear bands against the dark background). Proteolytic activity in stratum corneum extracts was also studied using substrates for (c) KLK7 (KHLY-pNA) or (d) trypsin-like KLKs (Ac-YASR-pNA). Substrate cleavage (detected by measuring the absorbance at 405 nm) is shown on the *y*-axis and time (hours) is shown on the *x*-axis. Data points represent the mean ± SEM from three experiments performed in triplicate, each experiment using a separate protein extract. Inhibitor treatments were also included to gauge the level of substrate cleavage that could be attributed to the target protease(s).

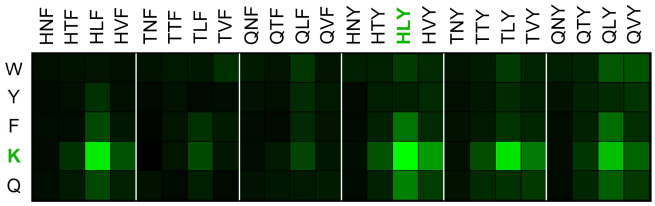
Figure 3. Exploring the contribution of separate KLK proteases to *ex vivo* corneocyte shedding. (a) Engineered inhibitors (indicated on the *x*-axis) were tested in *ex vivo* desquamation assays using 4 mm plantar stratum corneum biopsies, as described by Lundstrom and Egelrud (1988). The number of

detached corneocytes was quantified by measuring alkali soluble protein (bicinchoninic acid assay) and each data point represents a separate biopsy sample (protein content was measured in triplicate). Bars represent the mean ± SEM ($n \geq 6$), and data for each treatment were compared to no SFTI (control) using the Mann-Whitney test, with ** indicating $p < 0.01$ and *** indicating $p < 0.001$. SFTI-KLK5 (5 µM) was also compared to SFTI-KLK7 (1 µM) using the Mann-Whitney test ($p < 0.01$). Panel (b) shows data from (a) expressed as percentage inhibition, calculated by (% activity: no SFTI) – (% activity: SFTI treatment).

a



b **KLK7**



c **Chymotrypsin**